REVIEW ARTICLE

Structure and function of type II DNA topoisomerases

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INTRODUCTION

The faithful replication and segregation of chromosomes as part of the cell division cycle requires that topological changes be imposed upon cellular DNA. Interconversion of different topological forms of DNA is catalysed by DNA topoisomerases, a family of enzymes classified according to their catalytic mechanism of action. Type I enzymes introduce transient single-stranded breaks into DNA, pass an intact single strand of DNA through the broken strand, and then re-ligate the break. Type II enzymes, in contrast, make transient double-stranded breaks into one segment of DNA and pass an intact duplex through the broken DNA, before resealing the break. Topoisomerases are ubiquitous enzymes charged with the task of resolving topological problems which arise during the various processes of DNA metabolism, including transcription, recombination, replication and chromosome partitioning during cell division (reviewed by Wang, 1985; Sternglanz, 1989; Austin and Fisher, 1990; Osheroff et al., 1991; Gasser et al., 1992). As a result of performing this vital role, topoisomerases are necessary for the viability of all organisms from unicellular bacteria to humans. This intriguing family of enzymes has also aroused considerable interest since many antibacterial and antitumour drugs target topoisomerases and influence key steps in their catalytic cycle (reviewed in Beck and Danks, 1991; Capranico and Zunino, 1992; Fernandes et al., 1993; Pommier, 1993; Beck et al., 1993). In this review article, we shall concentrate on a discussion of the structure, mechanism of action and functions of the type II topoisomerase enzymes, particularly those from eukaryotic cells. However, it will be necessary, for comparison, to review briefly the type I enzymes.

DNA TOPOLOGY

The topological features of DNA that require definition are linking number, knotting and catenation. Linking number describes the number of times two DNA strands cross each other when projected onto a plane. Knotting and catenation are respectively the irreducible entanglement of a single DNA molecule, and the linking of two or more DNA molecules in which at least one strand of each duplex is in the form of a closed ring. As a consequence of the mechanistic differences between type I and type II topoisomerases, linking number changes catalysed by type I enzymes are in steps of one and those catalysed by type II enzymes are in steps of two. Linking number is always an integer and changes can be achieved only by breakage and resealing of either one or two DNA strands.

Linking number is dictated by two geometric functions, twist and writhe, which define different conformations which are adopted by closed-circular DNA in response to supercoiling changes. Twist is the extent to which the two complementary strands of DNA coil about the axis of the DNA helix, whereas writhe is the coiling of the helical axis in space. The sum of twist and writhe is the linking number of DNA, a topological parameter. DNA molecules differing only by changes in their specific linking number are termed topological isomers (topoisomers). By definition, the topoisomerase class of enzymes catalyse the interconversion of topological isomers of DNA. A comprehensive review of DNA topology has been published recently by Bates and Maxwell (1993).

REACTIONS CATALYSED BY DNA TOPOISOMERASES

The reactions catalysed by type II DNA topoisomerases are depicted in Figure 1. Of these reactions, only the relaxation of supercoiled DNA can also be catalysed by the type I topoisomerases. The reverse reaction, the introduction of supercoils into DNA, is specific for certain bacterial type II topoisomerases. Significantly, it is the type II enzymes alone which can knot/unknot and catenate/decatenate closed-circular DNA, because these reactions require the introduction of double-stranded breaks and the transport of a second duplex through the break. This process can also be achieved by type I enzymes under the special circumstance where at least one single-stranded nick or gap exists in the substrate molecule. Each of these reactions will be described in detail later.

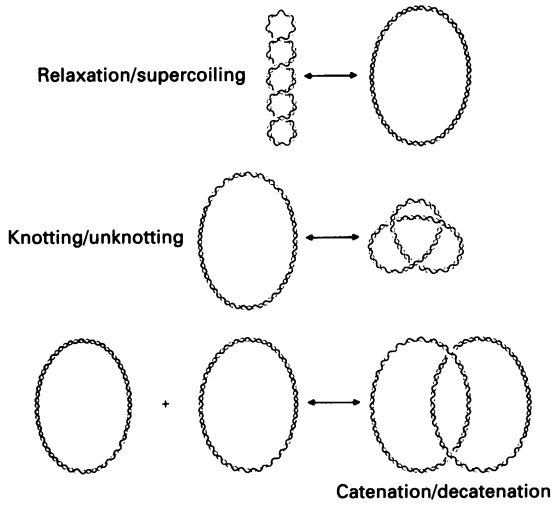

Figure 1 Reactions catalysed by type II DNA topoisomerases

All of these reactions can be catalysed by certain classes of type II enzymes; however, the promotion of DNA supercoiling is unique to bacterial DNA gyrases (for negative supercoiling, as shown) and reverse gyrases (for positive supercoiling, not shown). See text for details. This Figure is reproduced from Maxwell and Gellert (1986) with kind permission of Dr. A. Maxwell, University of Leicester, U.K., and Academic Press.

Abbreviations used: SAR, scaffold attachment region; MAP kinase, mitogen activated protein kinase.

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Table 1 A selection of the most extensively characterised type I and type II topoisomerases from different organisms

Some features of their catalytic mechanism of action, substrate specificity and cellular functions are shown in the right hand column.

Name	Class	Organism	Special Features
Topoisomerase I (ω protein)	I	*E. coli.*	Relaxes only negatively supercoiled DNA
Topoisomerase III	I	*E. coli.*	Involved in chromosome stability and plasmid segregation
DNA gyrase	II	*E. coli.*	Introduces negative supercoiling. Required for chromosome replication/segregation
Topoisomerase IV	II	*E. coli.*	No supercoiling activity. Required for chromosome segregation/cell division
Reverse gyrase	I	Various thermophilic bacteria	Introduces positive supercoiling
Topoisomerase I	I	*S. cerevisiae*	Relaxes negatively and positively supercoiled DNA
Topoisomerase II	II	*S. cerevisiae*	No supercoiling activity. Essential for chromosome segregation
Topoisomerase III	I	*S. cerevisiae*	Recombination function
Topoisomerase I	I	Human	Relaxes negatively and positively supercoiled DNA
Topoisomerase IIα	II	Human	No supercoiling activity. Function unknown
Topoisomerase IIβ	II	Human	No supercoiling activity. Function unknown

DNA TOPOISOMERASES FROM PROKARYOTIC AND EUKARYOTIC SPECIES

A selection of the most extensively studied topoisomerases is listed in Table 1, together with some of the reactions that they catalyse. Type I enzymes tend to be single chain polypeptides which act as monomers. In contrast, the type II enzymes show a multimeric structure containing at least two active sites, consistent with their ability to cleave simultaneously two strands of a DNA molecule. DNA gyrase of *Escherichia coli* comprises subunits encoded by the *gyr*A and *gyr*B genes and is a hetero-tetramer of A_2+B_2 configuration, while the eukaryotic topoisomerase enzymes, which can be viewed as single chain chimeras of the two DNA gyrase subunits, are consequently homodimeric in structure. A more detailed discussion of topoisomerase II protein structure can be found later in this article.

Bacterial topoisomerase I and II enzymes

E. coli contains two type I and two type II enzymes and the question of whether this represents specialisation or redundancy has been reviewed recently by Schmid and Sawitzke (1993). The two type I enzymes, topoisomerase I (encoded by the *topA/supX* gene) and topoisomerase III (encoded by *topB*) shared 24% amino acid identity over a 300 residue stretch and neither is essential for viability (reviewed in Wang, 1985; Sternglanz, 1989; Austin and Fisher, 1990). However, deletion of the *topA* gene requires that compensatory mutations occur in genes encoding one or other of the type II topoisomerases, DNA gyrase and topoisomerase IV, (Pruss et al., 1982; DiNardo et al., 1982; Raji et al., 1985; Oram and Fisher, 1992).

Analysis of mutants indicates that topoisomerase I is involved in transcription, acting alongside DNA gyrase to catalyse changes in supercoiling ahead of and behind the translocating transcription machinery. Mutants deficient in topoisomerase III show abnormalities in decatenation of ColE1-derived plasmids, consistent with the biochemical properties of topoisomerase III protein (DiGate and Marians, 1988). *TopB* mutants also show a defect in suppression of spontaneous deletions at sites of short sequence homology. Indeed, some *topB* mutants were originally isolated on the basis of their mutator phenotype (previously called *mutR*) (Schofield et al., 1992).

The two type II topoisomerases in bacteria are termed DNA gyrase and topoisomerase IV. DNA gyrase is encoded by the *gyr*A and *gyr*B genes, while topoisomerase IV is encoded by the *par*C and *par*E genes. DNA gyrase apparently has multiple roles *in vivo*, including general supercoiling homeostasis, the initiation phase of DNA replication, and chromosome partitioning (Steck and Drlica, 1984; Filutowicz, 1980; Kreuzer and Cozzarelli, 1979; reviewed in Wang, 1985; Sternglanz, 1989; Maxwell and Gellert, 1986). Prior to the discovery of topoisomerase IV, it was assumed that DNA gyrase was the sole decatenase in bacteria, consistent with the non-viability at the restrictive temperature of conditional mutants of the *gyrA* and *gyrB* genes. However, some mutations in topoisomerase IV subunit genes from both *E. coli* and *S. typhimurium* (*parC* and *parE*) also cause a conditional lethal phenotype (Kato et al. 1990, 1992; Schmid, 1990; Luttinger et al., 1991), suggesting that topoisomerase IV catalyses a stage in chromosome partitioning for which DNA gyrase cannot substitute.

A vivid demonstration of the role of type II topoisomerases in replicon segregation/partitioning came from an analysis of pBR322 plasmids in *S. typhimurium parC* and *parE* mutants (Adams *et al.*, 1992b). These strains, which are deficient in topoisomerase IV activity at the non-permissive temperature of 44 °C, accumulate high levels of supercoiled, catenated plasmids within 10–30 min of a shift to the non-permissive temperature. These catenated plasmids contain a variable number of nodes (points where the two duplex molecules cross when viewed in projection) ranging from 2 to > 32. However, as predicted from the two-stage model for the unlinking of replication intermediates (discussed in detail by Adams et al., 1992b), the catenanes were all right handed, parallel structures with the two duplexes helically wound around each other (so-called 'torus' catenanes). Figure 2 shows two examples of these replication intermediates which accumulate in the *parC* and *parE* mutants.

This discussion leaves open the question of the precise roles of the two bacterial type II topoisomerases. DNA gyrase almost certainly has a general role both in removing supercoils and in introducing negative supercoils into DNA, together with a more

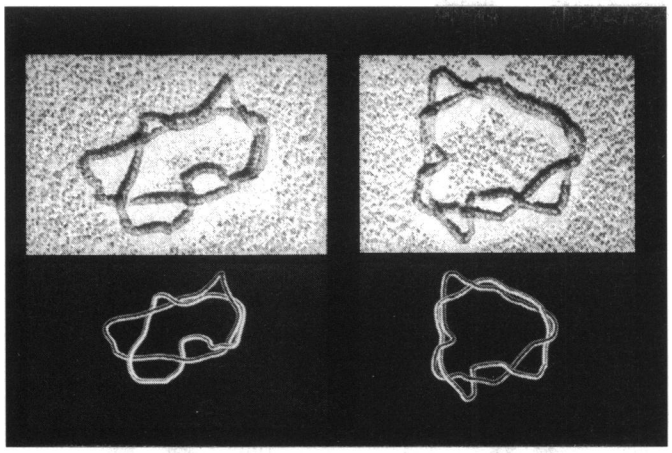

Figure 2 Electron microscopy of catenanes that accumulate in topoisomerase IV mutants

The pBR322 DNA was isolated from *parC* or *parE* mutants grown at the non-permissive temperature, coated in RecA protein and visualized by electron microscopy. Two examples of six-noded, right-handed, torus catenanes are shown, with drawings of each structure indicated below. This figure was reproduced from Adams et al. (1992b) with the kind permission of Dr. N. R. Cozzarelli, University of Berkeley, U.S.A. and Cell Press.

specialized role in removing knots and unlinking catenanes generated by recombination (Adams et al., 1992a). Topoisomerase IV, on the other hand, appears to fulfil the major role of dealing with catenanes generated by DNA replication (Adams et al., 1992b; Peng and Marians, 1993a,b). The mechanism(s) by which this specialization occurs is not clear, although one could imagine that the structure of the individual catenanes may differ, their cellular location may differ (the fact that topo IV is probably membrane-associated may be significant, see below) or interactions with other cellular proteins may differ. Any or all of these factors could impose specificity on the process of decatenation. In the light of these comments, it is perhaps surprising that co-expression of plasmid-encoded *gyrA* and *gyrB* genes can suppress lethality in *par* mutants, even when the chromosomally encoded *gyrA* and *gyrB* genes are intact (Kato et al., 1992). The precise reason for this 'gene dosage' effect of DNA gyrase expression in topoisomerase IV-deficient mutants is not clear at present.

The ParC protein (homologous to GyrA) is associated with the inner membrane in *E. coli* (Kato et al., 1992) and an attractive model for the role of topoisomerase IV is to act as an anchor (possibly in association with the product of the *parF* gene) for topologically constrained domains of DNA in a similar way to which eukaryotic topoisomerase II has been proposed to anchor looped domains of DNA to the nuclear matrix or scaffold (see below). Thus, in *E. coli*, the cell membrane may play a similar role to that of the chromosome scaffold in defining chromosome architecture (reviewed by Funnel, 1993).

Hyperthermophilic archaebacteria express a topoisomerase termed reverse gyrase (Kikuchi and Asai, 1984), which possesses both an unusual structure and an unusual enzymic mechanism of action. *E. coli* DNA gyrase and its bacterial homologues can introduce negative supercoils into DNA using the energy of ATP hydrolysis. Reverse gyrases, from such organisms as *Sulfolobus acidocaldarius*, use ATP to catalyse positive supercoiling of DNA. Reverse gyrase is unusual in that it not only represents a new class of 'gyrase' enzyme, but also because it is mechanistically more similar to the type I than the type II topoisomerases. Moreover, this enzyme is apparently unique among the type I topoisomerases in requiring ATP hydrolysis for action. The explanation for this puzzling array of properties came from sequence analysis of the gene encoding reverse gyrase from *S. acidocaldarius* (Confalonieri et al., 1993). The enzyme comprises two distinct domains; an N-terminal domain containing an ATP binding site and sequence motifs conserved in a family of DNA helicases, and a C-terminal domain with strong sequence homology to type I topoisomerases such as *E. coli* topoisomerases I and III. It would appear that this enzyme has been formed by fusion of two distinct DNA metabolizing enzymes to generate a chimeric species capable both of unwinding DNA and of altering DNA linking number. Helicase progression through duplex DNA can create positive supercoiling ahead of the translocating protein and negative supercoiling behind it. Assuming the type I topoisomerase moiety of reverse gyrase then specifically relaxes the negatively supercoiled DNA in the way which is characteristic of the bacterial type I topoisomerases, the substrate will progressively accumulate positive supercoils. Thus, this reaction is mechanistically completely distinct from classical supercoiling induced by DNA gyrases.

Yeast topoisomerases

Three distinct topoisomerases have been identified thus far from baker's yeast. The well characterized type I enzyme, termed topoisomerase I (*TOP1* gene product) is, like all of its eukaryotic counterparts, able to relax both negatively and positively supercoiled DNA (reviewed in Wang, 1985; Fisher et al., 1992; Gasser et al., 1992). Topoisomerase I is homologous to other type I topoisomerases and is not essential for viability. The functional similarity of yeast topoisomerase I to bacterial topoisomerase I enzymes is evident from the fact that the yeast *TOP1* gene will function in *E. coli topA* mutants as will the *topA* gene in yeast *top1* mutants.

The second type I topoisomerase identified in yeast is termed topoisomerase III (the *TOP3* gene product). This enzyme was identified via complementation of a hyper-recombination mutation, and deficiency in topoisomerase III confers a slow-growth phenotype (Wallis et al., 1989). The topoisomerase III protein is highly homologous to *E. coli* topoisomerase III protein and, to a lesser extent, to *E. coli* topoisomerase I. However, expression of *E. coli* topoisomerase I protein in yeast *top3* mutants complements the slow-growth defect, but not the hyper-recombination defect (Wallis et al., 1989). In agreement with its strong sequence similarity to bacterial type I topoisomerases, the yeast topoisomerase III protein only relaxes negatively supercoiled DNA (and then only very weakly) and forms a covalent link to the 5′ phosphoryl end of cut DNA (Kim and Wang, 1992). All other known eukaryotic type I topoisomerases form a phosphotyrosyl bond via the 3′ end of DNA breaks. The weak activity for the removal of DNA supercoils from covalently-closed molecules implies that topoisomerase III is unlikely to function in the general maintenance of chromosomal supercoiling in yeast. The hyper-recombination phenotype of *top3* mutants (Bailis et al., 1992) and the mutator phenotype of the most closely related homologue, *E. coli topB*, implies a role for these topoisomerase III enzymes in maintenance of genome stability.

In contrast to *E. coli* (and to human cells, see below) both budding and fission yeast appear to express a single type II topoisomerase, termed topoisomerase II. This enzyme differs from DNA gyrase in lacking supercoiling activity, but like DNA gyrase it is essential for cell viability. Conditional lethal strains with mutations in the *TOP2* gene encoding topoisomerase II are

incapable of traversing mitosis at the non-permissive temperature due to a failure to segregate replicated chromosomes (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986). Further discussion of the roles of eukaryotic topoisomerase II enzymes can be found later in this review.

The budding and fission yeast topoisomerase II protein sequences are highly similar to those of all known type II topoisomerases and, like their mammalian cell counterparts, consist of single polypeptide chains. There is an N-terminal region homologous to *E. coli* GyrB protein (and parE) and a C-terminal region with similarity to *E. coli* GyrA (and parC). A detailed discussion of the domain structure of type II topoisomerases is provided later in this review.

Human topoisomerases

Human cells express one type I and two type II topoisomerases. These enzymes appear to conform to the pattern set by their yeast counterparts both in terms of overall structure and substrate specificity. The gene encoding human topoisomerase I has been mapped to chromosome 20q12–13.2 and specifies a 4 kb mRNA (D'Arpa et al., 1988; Juan et al., 1988). Topoisomerase I is a 100 kDa monomeric protein with catalytic properties similar to those of the yeast type I enzyme. Roles for topoisomerase I in human cells are thought to include the initiation and elongation phases of DNA transcription and DNA replication (reviewed by Wang, 1985). Indeed, recent data indicate that topoisomerase I is a co-factor for transcription mediated by RNA polymerase II, affecting both basal transcription and activator-dependent transcription (Merino *et al.*, 1993; Kretzschmar *et al.*, 1993).

The two type II enzymes, designated topoisomerase IIα and topoisomerase IIβ, are the products of different genes (Drake et al., 1987, 1989; Chung et al., 1989; Austin and Fisher, 1990; Jenkins et al., 1992; Austin et al., 1993) encoded on chromosomes 17q21-22 (Tsai-Pflugfelder et al., 1988) and 3p24, respectively (Tan et al., 1992; Jenkins et al., 1992), and show distinct patterns of expression both during cell cycle transit and following oncogenic transformation of cells (Woessner et al. 1990, 1991). The topoisomerase IIα and β genes appear to have arisen via a relatively recent gene duplication event which included several flanking markers including the retinoic acid receptor α and β genes (Coutts et al., 1993).

The human topoisomerase IIα and β gene products are larger than their yeast counterparts due to an increase in the size of the C-terminal domain (see Figures 5 and 7 below). Although the C-terminal domain is likely to have some regulatory roles, the region proximal to the core catalytic domains is also probably important for some functional aspects such as dimerization or directing the protein to the cell nucleus (nuclear localization). Potential roles of the C-terminal domain of eukaryotic topoisomerase II enzymes are discussed later in this review.

THE CATALYTIC CYCLE OF EUKARYOTIC TOPOISOMERASE II

For topoisomerase II to catalyse changes in DNA topology requires a high-energy cofactor and Mg^{2+}. The catalytic cycle is extremely complex and precise mechanistic details of how DNA strands are transported through an enzyme-bound DNA gate are not yet available, although much progress has been made by the Osheroff, Wang and Westergaard groups in delineating certain features of the reaction. The catalytic cycle can be viewed as consisting of several discrete steps (Andersen et al., 1991; Svejstrup et al., 1993; reviewed in Osheroff et al., 1991), although in reality this is almost certainly an operational convenience. The catalytic cycle depicted schematically in Figure 3 is based upon models presented by Osheroff and colleagues, with certain modifications in the light of data presented recently by Roca and Wang (1992, 1994). In this Figure, the transported segment of duplex DNA is shown exiting from the interior of the enzyme through a gate located on the opposite side of the dimer to that of the entrance gate. This is shown in this way primarily for reasons of clarity. However, recent evidence supporting this so-

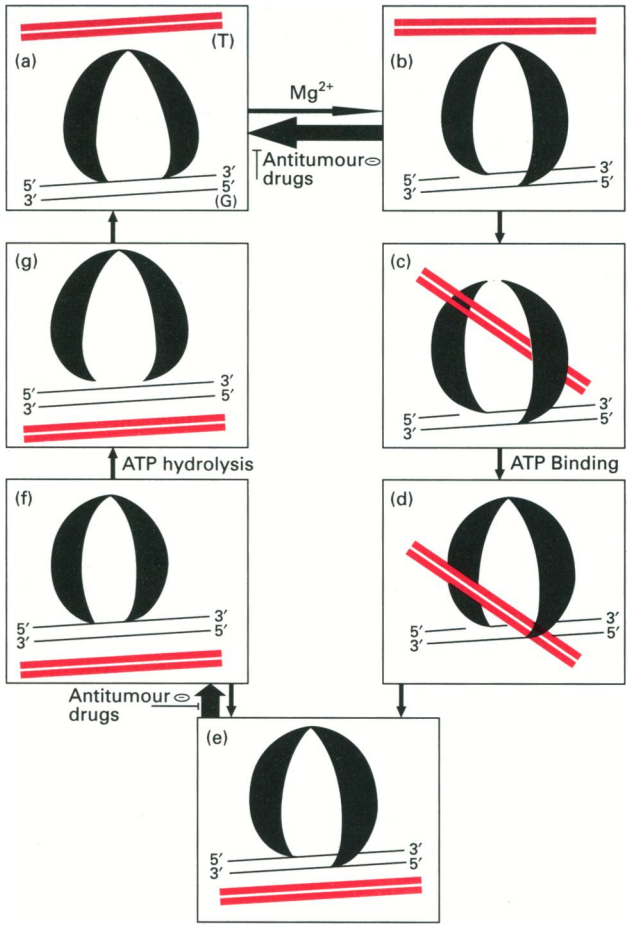

Figure 3 Schematic diagram of the catalytic cycle of topoisomerase II

The topoisomerase II dimer is shown as the black 'lobster claw' structure. The transported (or T) segment of duplex DNA is shown as two broad, parallel red lines, and the DNA which is cleaved by topoisomerase II and forms a transient gate (G-segment) is indicated as two thin, parallel black lines with the 5′ and 3′ orientation marked. The cycle begins at box a with recognition of the G-segment by topo II. The G- and T-segments of DNA are indicated in this box only. The enzyme then cleaves the G-segment, bonding covalently to the 5′ end of the nicked strands via the active-site tyrosine residues of each protomer (box b). This step requires Mg^{2+}. The enzyme then transports the intact T-segment through the gate in the G-segment in a strand passage reaction requiring ATP binding to close the enzyme clamp. The T-segment is shown entering the topoisomerase II dimer from the top (box c), passing through the protein (box d) and exiting at the bottom through the G-segment (box e). The cut G-segment is then re-ligated (box f). Following hydrolysis of ATP, enzyme turnover occurs (box g), permitting a further round of catalysis. It is not known precisely when ATP hydrolysis occurs, but it is likely to be following the strand passage reaction. Under processive conditions, step g, where the enzyme detaches from the G-segment of DNA, may effectively be omitted. The pre- and post-strand passage breakage and religation equilibrium reactions which can be affected by the cleavable complex-forming antineoplastic drugs are shown (between boxes a and b, and e and f, respectively). These drugs inhibit the religation steps, which are normally strongly favoured.

Table 2 Properties of the different classes of topoisomerase II inhibitors

The 'classical' cleavable complex(cc)-forming compounds, many of which are important antitumour drugs, are shown, together with novel inhibitors of the enzyme which in some cases antagonize cleavable complex formation by other drugs.

Drug Class	Examples	Special Feature
Acridines	Amsacrine (*m*-AMSA)	cc former
Actinomycins	Actinomycin D	cc former, also inhibits topo I
Anthracenediones	Mitoxantrone	cc former
Anthracyclines	Doxorubicin	cc former
Ellipticines	2-Methyl-9-OH ellipticinicum acetate	cc former
Benzisoquinolinediones	Amonafide	cc former
Epipodophyllotoxins	Etoposide, teniposide	cc former
Isoflavonoids	Genestein	cc former
bis-piperazinediones	ICRF159, 193	Inhibits DNA relaxation and antagonizes cc formation
Anthracenyl peptides	Merbarone	Antagonizes cc formation
Hexasulfated napthylurea	Suramin	Binds to topo II

called two gate mechanism has been provided [see Roca and Wang (1994) for a detailed description of the two-gate model].

The cycle begins with recognition of a DNA substrate by the topoisomerase II enzyme. The recognition of primary DNA sequence is not stringent, presumably because topoisomerases need to monitor the tertiary structure of DNA in order to act at any point within the genome where topological problems exist. Topoisomerase II has affinity for both intra- and inter-molecular DNA crossovers even in the absence of Mg^{2+}. However, Roca et al. (1993) demonstrated that this binding is not apparently directly related to catalysis of DNA strand passage.

Once bound to its substrate, a pre-strand passage DNA breakage and religation equilibrium is established, with the religation step favoured. Both strands of the DNA molecule are broken with the formation of a 4 bp staggered gate containing a topoisomerase protomer bound covalently via a O^4-phosphotyrosyl bond to the 5′ end of each broken strand. This reaction requires Mg^{2+}. An intact double-stranded DNA segment is then passed through the gate in a reaction requiring binding of ATP, but not its hydrolysis. After strand passage, a second breakage–religation equilibrium is established which again strongly favours religation. Following successful sealing of the break, ATP hydrolysis occurs to facilitate enzyme turnover and the re-initiation of a subsequent cycle.

Elegant experiments by Roca and Wang (1992) have provided an important insight into the mechanism of the topoisomerase II reaction. These authors made use of a filter binding assay (Thomas et al., 1979) to demonstrate that in the presence of the non-hydrolysable ATP analogue, AMPPNP (and presumably ATP itself, although this could not be tested directly), topoisomerase II undergoes a conformational change such that it can bind to linear, but not to circular DNA. This preference is not due to a specific interaction with the ends of the linear fragment but rather is due to an intrinsic property of the enzyme. If the linear DNA is subsequently circularized, it forms a stable catenane with the topoisomerase II protein. However, if the enzyme is preincubated with circular DNA prior to the addition of the ATP analogue, the enzyme can capture the circular DNA in a stable complex which can only be released by denaturation of the protein with detergents. It is likely that this complex involves the encircling of the DNA by topoisomerase II, in the form of a protein clamp. This paradigm of proteins clamping around DNA molecules also extends to some DNA polymerases (reviewed by Kuriyan and O'Donnell, 1993). Presumably, once this protein clamp is in place, only linear DNA can subsequently thread through the annulet. In the case of topoisomerase II, closure of the jaws of the protein clamp is apparently dependent on the presence of AMPPNP and occurs irrespective of whether DNA is present or not. Transport of one DNA segment (denoted the T-segment in Figure 3), through another segment containing a gate (the G segment) is possible only if the T segment enters the open complex containing both topoisomerase II and a prebound G-segment. The strand passage event occurs upon the addition of AMPPNP, which allows the concomitant closure of the clamp. *In vivo*, the binding of ATP would allow the reversible opening and closing of the protein clamp. If two DNA duplexes sequentially enter the enzyme when the jaws of the clamp are open, the strand passage event can occur. Consistent with this model is the observation that topoisomerase II undergoes a conformational change upon the binding of AMPPNP. This change is evidenced by the replacement of a Staphylococcal V8 protease cleavage site at Glu-410 by an alternative site at Glu-680 upon the binding of AMPPNP (Lindsley and Wang, 1991, 1993).

Lindsley and Wang (1993) have recently shown that the coupling between ATP hydrolysis and strand passage is not tight, suggesting that some ATP may be hydrolysed without a productive strand passage event occurring.

Effects of antineoplastic drugs on the catalytic cycle of eukaryotic topoisomerase II

A wide variety of antitumour drugs have been shown to influence the catalytic cycle of topoisomerase II (for reviews see Osheroff et al., 1991; Pommier, 1993; Beck et al., 1993). Intercalating agents, such as doxorubicin, mitoxantrone and mAMSA, as well as the non-intercalating epipodophyllotoxins, etoposide and teniposide, stabilize a reaction intermediate termed the 'cleavable complex' consisting of a topoisomerase II protomer bound covalently to each 5′ end of the broken DNA molecule. These drugs can act at at least two points in the topoisomerase II catalytic cycle. Both the pre- and post-strand passage equilibria are disturbed as a result of a decrease in the enzyme's apparent first order rate constant for religation (Figure 3).

A selection of some of the best known 'cleavable complex' forming topoisomerase II poisons are shown in Table 2, together with certain non-cleavable complex-generating compounds,

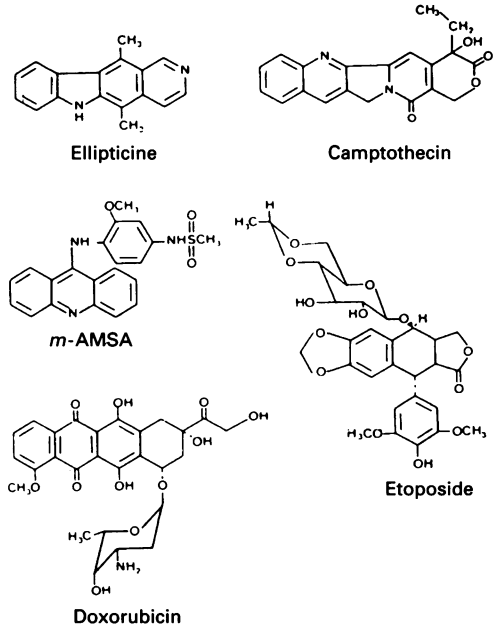

Figure 4 Structures of different classes of topoisomerase poison

The single topoisomerase I inhibitor shown is camptothecin. The four topoisomerase II inhibitors are representative of different drug classes; the acridines (*m*-AMSA), the anthracyclines (doxorubicin), the ellipticines (ellipticine) and the epipodophyllotoxins (etoposide, also called VP16).

many of which are used as reagents for the study *in vitro* of topoisomerase II action. The structures of representative members of the best known classes of cleavable complex-forming topoisomerase I and II poisons are shown in Figure 4. Of these, the anthracyclines (including doxorubicin and daunomycin) and the epipodophyllotoxins (etoposide and teniposide; also called VP16 and VM26) are particularly noteworthy as they form important constituents of curative therapies for lymphomas, testicular and breast cancers, and certain leukaemias. These drugs are also important in producing prolongation of life in patients with small cell cancer of the lung (for reviews, see Myers, 1993; Wozniak and Ross, 1993).

Antitumour drugs have proved useful as laboratory reagents for probing the mechanism of topoisomerase II action and, in particular, whether the enzyme displays sequence selectivity during DNA binding/cleavage. Using the cleavable complex-generating drugs, it is possible to trap the enzyme in the form covalently complexed with nicked DNA, thus facilitating cleavage-site mapping. It is clear that the choice of DNA cleavage sites is influenced both by the enzyme itself and by the particular class of drug used. The bases flanking the cleavage sites are similar regardless of the class of inhibitor present (and thus enzyme-specific), while the preferred base immediately 5′ or 3′ to the cleavage site in each case is specific for the drug used; examples being a 5′ cytosine for mitoxantrone or a 5′ adenine for *m*-AMSA, and a 3′ thymine for ellipticine or a 3′ cytosine for etoposide (Capranico et al., 1990; Fosse et al., 1991; Pommier et al., 1991; Freudenreich and Kreuzer, 1993). Moreover, there is significant base symmetry around the cleavage site. These data suggest two things. First, that the individual protomers of the topoisomerase II homodimer interact in a similar way with each strand of the recognition sequence. Secondly, that the drug must be bound at or very near to the site of DNA cleavage. Indeed, it has been suggested that the drug actually stacks in the DNA at the site of cleavage alongside the preferred base for that particular drug (see Pommier, 1993). Consistent with these data, Freudenreich and Kreuzer have shown that a photoreactivatible derivative of *m*-AMSA can be cross-linked to one of the residues in the bp 5′ to two cleaved phosphodiester bonds (K. Kreuzer, personal communication).

Recent work by Roca et al. (1994) has shown that ICRF-193, a member of the bisdioxopiperazine class of drugs, inhibits topoisomerase II via a novel mechanism. Specifically, the DNA relaxation activity of the enzyme is inhibited, but this can be reversed by removal of the drug. Using the filter binding assay described above, these authors showed that in the presence of ATP, ICRF-193 locks the enzyme in a salt-stable conformation, incapable of binding circular DNA. The formation of this salt-stable complex requires ATP for its establishment but not for its maintenance. Protease digestion studies were used to show that the ICRF193-inhibited enzyme was trapped in a conformation thought to be analagous to the ATP-bound, 'closed-clamp' form of the enzyme (Roca and Wang, 1992; described above). Thus, this drug appears to act by blocking the interconversion between the open- and closed-clamp forms of the enzyme.

FUNCTIONS OF TOPOISOMERASE II

Segregation of chromosomes during mitosis and meiosis

The major functions of topoisomerase II are summarized in Table 3 and discussed in detail below. A number of studies have shown that the gene encoding topoisomerase II (*TOP2*) is essential for viability in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Goto and Wang, 1984; Uemura and Yanagida, 1984). Analysis of temperature sensitive *top2* mutants of *S. cerevisiae* (DiNardo et al., 1984; Holm et al., 1985; Rose and Holm, 1993) and *S. pombe* (Uemura and Yanagida, 1986; Uemura et al., 1987), has demonstrated that topoisomerase II is required for chromosome segregation at mitosis and meiosis, during which the enzyme is thought to untangle intertwined daughter strands created by DNA replication. In support of this, *S. cerevisiae* and *S. pombe top2* mutants accumulate multiply-intertwined, catenated dimers derived from newly replicated circular plasmids (DiNardo et al., 1984; Uemura and Yanagida 1986). This failure to segregate intertwined DNA molecules leads eventually to cell death as the cell attempts to divide at mitosis (Holm et al., 1985; Uemura and Yanagida, 1986). The requirement for topoisomerase II during mitosis has been confirmed by the ability of the spindle poison nocodazole to rescue cell viability at the non-permissive temperature in conditional

Table 3 Roles for eukaryotic topoisomerase II, as defined by analysis of yeast *top2* mutants

Role	Manifestation of top2 mutation
Mitosis: Chromosome segregation	Aberrant mitosis, cell death
Mitosis: Chromosome condensation	Arrested cells have precondensed chromatids
Meiosis	Arrest in meiosis I (viability retained)
Recombination suppression	Hyper-recombination in rDNA cluster

top2 mutants (Holm et al., 1985). In *S. pombe top2* mutants, uncondensed chromosomes are transiently pulled into filamentous structures by the mitotic spindle, which ultimately fails to separate them. It is this specific defect which is thought to disrupt segregation.

The work of Holm et al. (1989) suggests that the disruption of segregation in *S. cerevisiae top2* mutants leads to chromosome loss as well as chromosome breakage (which was also shown to result from the absence of topoisomerase II). Apparently, in the absence of active topoisomerase II, small chromosomes (with arms less than approximately 250 kb) can resolve intertwinings through simple unravelling of their ends, while larger chromosomes are more inclined to fragment (Spell and Holm, 1994).

The inhibition of topoisomerase II in living *Drosophila* cells provides a vivid illustration of the role of this enzyme in the segregation of chromosomes at mitosis in a multicellular organism. Microinjection of either antibodies against topoisomerase II or the drug VM26 into *Drosophila* embryos can inhibit the separation of chromosome daughter sets at anaphase (Buchenau et al., 1993).

The segregation of recombined chromosome in meiosis I requires DNA topoisomerase II (Rose et al., 1990). Recently, it has been demonstrated that *top2* mutants show a cell-cycle arrest phenotype at the late pachytene stage of meiosis I, before the establishment of the spindle (Rose and Holm, 1993). This result suggests that meiotic cells differ from mitotic cells in having a checkpoint mechanism for ensuring that sufficient active topoisomerase II is present to effect chromosome segregation. If this requirement is not fulfilled, a checkpoint-mediated cell division arrest results, preventing attempted chromosome segregation which would prove lethal for the cell.

Topoisomerase II and chromatin condensation

Topoisomerases may help accomplish the formidable tasks of condensing DNA into chromatin and further condensing this chromatin into mitotic chromosomes. Topoisomerases are also likely to be required for the decondensation of these structures.

Several lines of evidence suggest a role for topoisomerase II in mitotic chromosome condensation. In a conditional *top2* mutant of *S. pombe*, chromosomes are not hypercondensed at the non-permissive temperature (Uemura et al., 1987). It is interesting to note, however, that *S. cerevisiae top2* mutants show no such abnormality in chromosome condensation at meiosis (Rose and Holm, 1993), although the degree to which chromosomes are condensed during cell division is generally greater in fission than in budding yeast cells. Whether these data truly reflect a difference between either the mechanics of chromosome condensation during meiosis and mitosis, or of the role(s) of topoisomerase II in these processes, remains to be established.

Treatment of chromatin with the drug VM26 inhibits artificial chromosome condensation *in vitro* in extracts from both somatic cells and *Xenopus* oocytes (Hirano and Mitchison, 1993). A similar result is evident following immunodepletion of topoisomerase II (Wood and Earnshaw, 1990; Adachi et al., 1991; Hirano and Mitchison, 1993). More recently, the non-cleavable complex forming drug ICRF-193 was shown to inhibit the decondensation as well as the condensation of chromosomes *in vivo* (Ishida et al., 1993)

Those working with topoisomerase II have been aware of its propensity to aggregate *in vitro*. One model suggests that the aggregation of topoisomerase II when attached to DNA helps to mediate chromosome condensation. How this would be regulated is not clear; however, one could imagine that cyclical modification of the enzyme, such as by phosphorylation and dephosphorylation, might control chromosome condensation and decondensation. However, it should be noted that topoisomerase II can be extracted from mitotic chromosomes under conditions where other mitotic phosphoproteins (recognized by the MPM-2 antibody) remain associated with the scaffold (Hirano and Mitchison, 1993).

Topoisomerase II in DNA replication and transcription

DNA tracking processes such as replication and transcription generate positive supercoils ahead of the tracking complex and negative supercoils behind it (Liu and Wang, 1987). This effect may only be transient if the diffusion of supercoils is allowed to have a cancelling effect. However, if the template, polymerase or transcript is anchored to some structure (see Cook, 1989, 1991 for reviews), or if there are other barriers to rotational diffusion of the transcription or replication complex such as the viscosity of the nuclear milieu, stable accumulation of supercoiled domains can occur. This has been termed the 'twin domain model' of supercoiling (Liu and Wang, 1987; reviewed by Wang and Lynch, 1993). Since negative supercoiling enhances transcriptional initiation (Parvin and Sharp, 1993) and, conversely, positive supercoiling inhibits transcription (Gartenberg and Wang, 1992), expression and replication of genes lying ahead of a tracking complex would seem to be dependent on the presence of a DNA relaxing activity in cells. This 'swivelase' function could equally be provided by topoisomerase I or II.

Owing to the redundancy of swivelase activities, either topoisomerase I or topoisomerase II is sufficient to stop the buildup of supercoils during the elongation phase of DNA replication in yeast. In the absence of topoisomerase I, however, chain extension is somewhat delayed in the early stages of replication suggesting that topoisomerase I may be important for at least this role (Kim and Wang, 1989a). Mutation of topoisomerase II alone, on the other hand, has little effect on the rate of chain elongation, supporting the idea that topoisomerase I is the normal replication swivel, at least in budding yeast (Kim and Wang, 1989a). Indeed, topoisomerase I seems to associate preferentially with the replication fork region (reviewed by Wang and Liu, 1990). It appears that topoisomerase II is only crucial for the separation of intertwined, newly replicated chromosomes and the decatenation of extrachromasomal rings during the later phases of DNA replication. This role has been demonstrated by the finding that catenated dimers and late Cairns-type replication intermediates accumulate during the replication of SV40 DNA *in vitro* when topoisomerase II is inhibited or depleted from extracts (Sundin and Varshavsky, 1981; Yang et al., 1987; Richter and Strausfeld, 1988; Ishimi et al., 1992) or inhibited *in vivo* (Richter et al., 1987; Snapka, 1986).

The most recent studies using the non-cleavable complex-forming drug ICRF-193 to probe the role of topoisomerase II in the late stages of DNA replication (Ishimi et al., 1992; Clarke et al., 1993) are easier to interpret than those utilizing drugs such as VP16 or VM26, because the formation of cleavable complexes can block chain extension at an early stage. Thus the presence of abortive replication intermediates could in principle be due to a physical blockage of replication and not to a lack of enzymic resolution. Indeed, the cleavable complex-forming drugs kill cells primarily in S-phase, unlike ICRF-193, which mimics the phenotype of yeast *top2* mutants by causing cell death during the G2/M phase. The above data suggest that topoisomerase II is not required for the early stages of replication but is necessary to facilitate resolution of late replication intermediates, as well as to

decatenate catenanes (in the case of SV40 or 2μ plasmid replication).

Topoisomerase II and recombination

Mutation of the topoisomerase II gene (or the topoisomerase I gene) in *S. cerevisiae* greatly increases recombination levels in the rDNA cluster (Christman et al., 1988). Indeed, in a *top1 top2* double mutant strain, the rDNA gene cluster is particularly unstable, tending to be excised as extrachromosomal DNA rings (Kim and Wang, 1989b). Augmenting the level of topoisomerase II or topoisomerase I activity in such a *top1 top2* strain restores genome stability, demonstrating the direct involvement of these enzymes in suppressing abnormal recombination (Kim and Wang, 1989b). It has been postulated that these mechanistically dissimilar topoisomerases might act via their common relaxation activities to avoid the intertwining of inappropriately paired DNA strands which might otherwise form due to the local accumulation of supercoils through transcription (reviewed by Kanaar and Cozzarelli, 1992; Droge, 1994). Highly transcribed repetitive sequences such as the rDNA cluster would be expected to be particularly susceptible to such an effect (Wang et al., 1990). Indeed, the recombination-stimulating sequences in the rDNA cluster correspond to regions involved in regulation of transcription by RNA polymerase I (Voelkel-Meiman et al., 1987). It should be noted, however, that the presence of topoisomerase I relaxing activity was not able to block a resolvase reaction *in vitro*, which is dependent upon gene transcription, and thus presumably upon the supercoiling that this generates (Droge, 1993). This may nevertheless simply reflect the inability of topoisomerase I to relax local supercoils at a sufficient rate. The level of mitotic recombination is slightly elevated when conditional yeast *top2* mutants are shifted to the restrictive temperature (Holm et al., 1989). This is thought to be an indirect consequence of the increased chromosome breakage observed in the absence of active topoisomerase II.

The involvement of eukaryotic topoisomerase II in illegitimate recombination is controversial. While certain eukaryotic topoisomerase II enzymes can mimic the phage T4 homologue in stimulating such recombination *in vitro*, evidence is lacking for an involvement *in vivo* (reviewed by Ikeda, 1990).

Is topoisomerase II a chromosome scaffold protein?

Several lines of evidence support the notion that topoisomerase II plays a structural role in chromosomes (reviewed in Gasser et al., 1989; Roberge and Gasser, 1992). Specifically, it has been suggested that topoisomerase II might act as an anchor joining specific DNA sequences found at the base of chromosomal loops, termed scaffold attachment regions or SARs, to the nuclear scaffold (reviewed by Gasser and Laemmli, 1987). This hypothesis is supported by several observations. First, a consensus sequence for topoisomerase II DNA cleavage *in vitro* is frequently found in SAR sequences (Udvardy et al., 1985). Indeed, topoisomerase II has been shown by competition experiments to interact *in vitro* with DNA containing a SAR sequence (Adachi et al., 1989; Kas et al., 1989). It should be noted, however, that DNA sequence specificity is not the primary determinant of scaffold attachment (Udvardy and Schedl, 1991). Secondly, topoisomerase II is the major non-histone protein present in standard preparations of the nuclear scaffold (Earnshaw and Heck, 1985; Gasser et al., 1986). Thirdly, topoisomerase II can be isolated from a nuclear protein complex containing chromosome scaffold protein-2 (Ma et al., 1993). Finally, immunohistochemical experiments have suggested that topoisomerase II is specifically localized to axial filaments in mitotic chromosomes (Earnshaw and Heck, 1985; Gasser et al., 1986) and in meiotic chromosomes (Moens and Earnshaw, 1989; Klein et al., 1992). These experiments are controversial, however, since they were carried out on chromosomes which had been swollen (albeit gently), potentially allowing artifactual interactions to occur. Another objection is that scaffold/matrix attachment sites are operationally defined as the chromosomal fraction resistant to certain histone extraction procedures (see Cook, 1989 and Cook, 1991, for reviews). Thus, the conditions used to isolate matrix proteins may cause artifactual aggregation of topoisomerase II. It may now be possible to test whether putative SARs are able to anchor DNA *in vivo* using a newly devised assay based upon site-specific recombination in yeast cells (Gartenberg and Wang, 1993).

According to recent immunolocalization work using *Xenopus* extracts (Hirano and Mitchison, 1993) and living *Drosophila* embryos (Swedlow et al., 1993), topoisomerase II is not specifically associated with axial filaments but rather is distributed uniformly throughout chromosomes. Moreover, inhibition of topoisomerase II was shown to have little effect on the morphology of chromosome assembly *in vitro* using *Xenopus* extracts (Hirano and Mitchison, 1993). In a separate study, Fischer et al. (1993) showed that topoisomerase II is absent from *Xenopus* meiotic (lampbrush) chromosomes. Thus, the most recent and direct results suggest that topoisomerase II may not play a specific scaffolding role.

STRUCTURAL AND FUNCTIONAL DOMAINS OF TOPOISOMERASE II

When the sequences of various eukaryotic type II topoisomerases are compared, three discrete domains can be discerned (Figure 5); an N-terminal region homologous to the B subunit of bacterial gyrase (GyrB), a central region homologous to the GyrA subunit, and a C-terminal region characterized by clusters of charged amino acids (see Lynn et al., 1986; Wyckoff et al., 1989; Huang, 1990; Jenkins et al., 1992; Austin et al., 1993; Caron and Wang, 1994 for discussion of topoisomerase II homologies). However, it is interesting to note that the topoisomerase II enzyme encoded by T-even phages lacks the non-conserved C-terminal region (Huang, 1990). The functional conservation of these enzymes is remarkable. While they share only about 50% sequence homology, the *S. pombe*, *Drosophila*, mouse and human topoisomerase IIα genes can complement a *S. cerevisiae top2* mutation (Uemura et al. 1987, Wyckoff and Hsieh, 1988; Adachi et al., 1992; Wasserman et al., 1993). We have shown that the human β isozyme can also complement a *top2* mutation (J. R. Jenkins, S. Jensen and I. D. Hickson, unpublished work). Moreover, the conditional overexpression of a *Drosophila* topoisomerase II enzyme can complement the drug sensitivity of a mutant topoisomerase II enzyme in mammalian cells (Eder et al., 1993).

Susceptibility to protease digestion also defines at least three regions of the topoisomerase II polypeptide. These regions probably reflect distinct folded domains, since the cleavage sites are well conserved between enzymes from different species (Reece and Maxwell, 1989; Lindsley and Wang., 1991; Shiozaki and Yanagida, 1991; Lee and Hsieh, 1994). The N-terminal fragment contains a consensus for an ATP-binding site, has the capacity to hydrolyse ATP *in vitro* (Ali et al., 1993) and mutations within this domain abolish the ATPase activity of the *E. coli* GyrB protein and the yeast topoisomerase II enzyme (Jackson and Maxwell, 1993; Lindsley and Wang, 1991). The structure of this region in GyrB has been solved by X-ray diffraction analysis and illustrates the presence of a 220-residue binding-pocket for ATP

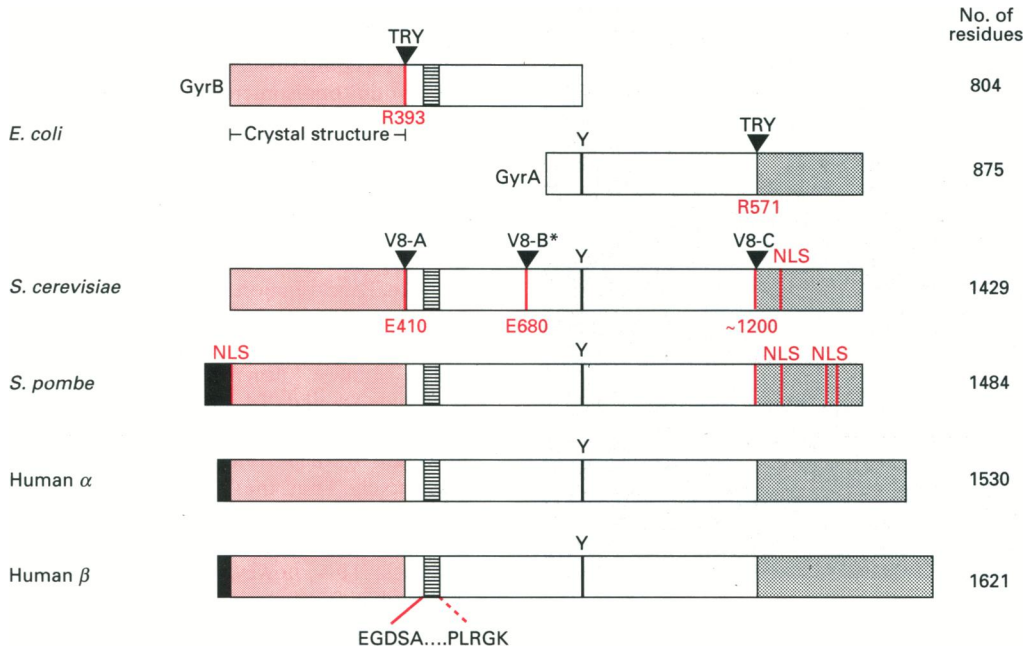

Figure 5 Domain structure of topoisomerase II enzymes from prokaryotes and eukaryotes

A schematic representation of the domain structure of one prokaryotic (*E. coli* DNA gyrase) and four eukaryotic topoisomerase II enzymes is shown. DNA gyrase consists of two polypeptides, GyrB (left) and GyrA (right), while all eukaryotic forms are single polypeptide chains. The number of amino acid residues in each protein is indicated on the right. The N-terminal ATP binding domain is shown in light red. The ATP-binding region of *E. coli* GyrB for which X-ray crystallographic data exists is indicated. The central DNA binding and DNA breakage/reunion domain is an open box. The C-terminal domain is grey. Additional notable features are: (1) A short N-terminal extension of unknown function in the *S. pombe* and human enzymes shown with black; (2) A horizontally hatched box denoting two amino acid motifs: EGDSA ... PLRGK (single letter amino acid code) defining a conserved region in all type II topoisomerases; (3) The active-site tyrosine residue denoted by a black vertical line labelled Y; (4) putative nuclear localization signals denoted by red vertical lines labelled NLS. The domain boundaries have been defined by reference to protease-sensitive sites within the *E. coli* or *S. cerevisiae* proteins. The DNA gyrase subunits each contain a preferred trypsin cleavage site, following arginine 393 (R393) in GyrB and arginine 571 (R571) in GyrA, marked by black arrowheads labelled TRY. The *S. cerevisiae* enzyme has two preferred sites for V8 proteinase digestion, after glutamate 410 (E410) and around residue 1200, marked by the black arrowheads labelled V8-A and V8-C, respectively. The third V8 cleavage site (V8-B*, at glutamate 680) is conditional upon ATP binding to the enzyme (described in the text).

(Wigley et al., 1991). This domain dimerizes in the presence of a non-hydrolysable ATP analogue (Figure 6) and must therefore contain sequences which direct protein–protein interactions. Several linker insertion mutations in the corresponding region of the *Drosophila* enzyme abolish activity (Lee and Hsieh, 1994).

A particularly interesting internal cleavage site in the *S. cerevisiae* enzyme corresponds approximately to the junction between the *E. coli* GyrB and GyrA protein homologies, and is dependent on the presence of the non-hydrolysable ATP analogue AMPPNP. This so-called 'conditional' site B (see Figure 5) is cleaved in preference to the most N-terminal site (designated site A) in the presence of AMPPNP (Lindsley and Wang, 1991). This phenomenon is thought to reflect a conformational change induced by the binding of ATP to the enzyme. Between protease sensitive sites A and B is a region implicated in DNA binding and/or catalysis, which is highly conserved among type II topoisomerases.

There are three amino acid motifs in the central region of the enzyme (in the one letter amino acid code: EGDSA, PL(R/K)GK(I/L/M)LN and IM(T/A)D(JQ/A)D; see Figure 5) which are conserved in all topoisomerase II proteins sequenced to date. Sequence comparisons suggest that the EGDSA and PLRGK motifs may correspond to loops found in the structure of the $\gamma\delta$ resolvase, a protein to which the topoisomerases is related (Caron and Wang, 1993). Linker insertions near these motifs reduce the activities of the *Drosophila* enzyme (Lee and Hsieh, 1994). Several mutations within the PLRGK box also affect resistance to inhibition by antineoplastic drugs, implicating this region in the binding of drugs to the protein/DNA complex (reviewed in Caron and Wang, 1993, 1994). The active site tyrosine which covalently binds to DNA is found C-terminal to the 'conditional' protease site B (Horowitz and Wang, 1987, Worland and Wang, 1989).

The region of the eukaryotic topoisomerase II protein most sensitive to proteases defines the beginning of the C-terminal region (Reece and Maxwell, 1989; Lindsley and Wang, 1991; Shiozaki and Yanagida, 1991; Lee and Hsieh, 1994). However, the extreme C-terminus of the enzyme is not sufficiently well conserved to allow the identification of large structural motifs (Caron and Wang, 1993, 1994) The putative roles of the C-terminal region is discussed below.

INTERACTIONS BETWEEN TYPE II TOPOISOMERASES AND OTHER NUCLEAR PROTEINS

There is mounting evidence that type II topoisomerases are able to associate with other proteins. For example, Earnshaw et al. (1985) and Gasser et al. (1986) have provided immunocytochemical evidence that topoisomerase II is a major component of the nuclear scaffold, a conclusion which presumably implies interaction with other scaffold proteins (discussed above). Although it seems likely that topoisomerase II participates in DNA replication, transcription and recombination as part of a multienzyme complex, direct evidence for specific protein–protein interactions is scarce. However, topoisomerase II has been shown to be part of a large complex isolated from yeast cells which

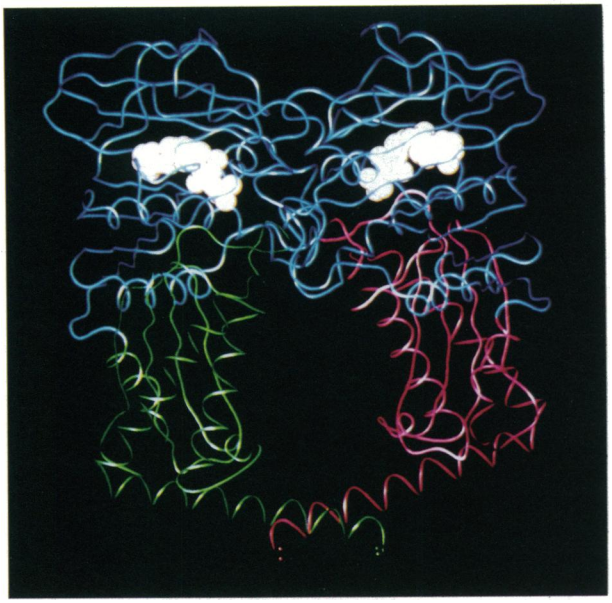

Figure 6 Structure of the 43 kDa ATP-binding domain of *E. coli* GyrB protein

The protein backbone is shown as coloured ribbons. In the presence of a non-hydrolysable analogue of ATP (AMPPNP), the protein dimerizes with each protomer binding one AMPPNP molecule (shown as the white space-filling model). Each protomer is depicted as comprising two domains: an N-terminal AMPPNP binding domain (blue in each case) and a C-terminal domain (shown as green in one protomer and red in the other). Contacts are made between the C-terminal domains of the individual protomers, forming a hole of diameter approximately 20 Å running through the dimer. Because the recombinant GyrB proteins were truncated at their C-termini, the precise geometry of the protomer contacts to generate the hole are still unclear. Figure provided by courtesy of Dr. Dale Wigley, University of Oxford, U.K.

allows replication of the 2μ plasmid *in vitro* (Jazwinsky and Edelman, 1984) and of a complex that binds 5′ to an immunoglobulin gene promoter (Webb et al., 1993).

A DNA-binding protein involved in cellular proliferation has recently been isolated from HeLa cells. Intriguingly, one subunit of this heterodimer contains the PLRGK motif common to all type II topoisomerases (Zhang et al., 1992).

The putative leucine zipper region found in many eukaryotic topoisomerase II enzymes (Zwelling and Perry, 1989; Caron and Wang, 1994) may be important either for dimerization of the individual topoisomerase II protomers or for interactions with other proteins. Moreover, yeast *top2* mutations conferring a cold sensitive phenotype have been mapped to between the conserved leucine residues (Thomas et al., 1991). Leucine zippers are found in a wide range of eukaryotic nuclear enzymes, including several transcription factors, such as *fos*, *jun*, and *Creb*, where they direct the formation of homo- and hetero-dimers. However, site-directed mutagenesis studies of the zipper region in the human topoisomerase IIα protein failed to reveal any role for the leucine repeat sequence in topoisomerase II protein dimerization (Kroll et al., 1993). These authors did, however, find that a large region of the topoisomerase IIα protein (from amino acids 857 to 1000) is important for association with other proteins, such as CREB. Whether this region adjacent to the leucine zipper also plays a role in protein–protein interactions between individual topoisomerase IIα protomers is not clear at this stage. However, we have recently found evidence of a novel, putative helicase interacting with this region *in vivo* (P. M. Watt and I. D. Hickson, unpublished work).

ROLES FOR THE C-TERMINAL REGION OF EUKARYOTIC TOPOISOMERASE II ENZYMES

Sequence comparisons indicate that the C-terminal region of eukaryotic topoisomerase II proteins has diverged significantly between species. Indeed, the two human topoisomerase II isoenzymes show only around 35% amino acid identity in this region. This contrasts sharply with the remarkably high level of sequence conservation within the ATPase and breakage/reunion domains of topoisomerase II enzymes right across the evolutionary scale from *E. coli* to humans. For example, the ATPase and breakage/reunion domains of the human β isoenzyme are 51% and 48% similar to the equivalent domains of *E.coli* GyrB and GyrA proteins, respectively (Jenkins et al., 1992).

The smallest known topoisomerase II enzymes, those from bacterial T2 and T4 phage, are essentially composed of an ATPase domain and a breakage/reunion domain, although these units are formed from two and three separate polypeptides, respectively. Thus, the C-terminal region present in the eukaryotic topoisomerase II enzymes is extremely unlikely to contain residues required for formation of the catalytic active sites of the enzymes. This, however, does not imply that this domain is dispensible for function, as will be outlined below. For the purpose of this discussion, we shall define the C-terminal region as comprising approximately 25% of the protein, including the residues implicated in protein–protein interactions (see above). Thus, this region includes approximately 100 additional residues not present in the C-terminal domain defined above on the basis of partial proteolysis of the enzyme.

A number of different approaches, with different eukaryotic topoisomerase II enzymes, have been adopted to ascertain the role(s) of the C-terminal region. We shall discuss three potential roles: nuclear localization, dimerization and regulation of enzymic activity (summarized in Figure 7).

Nuclear localization

There are a number of potential nuclear localization signals (reviewed by Dingwall and Laskey, 1991) throughout the sequences of eukaryotic topoisomerase II enzymes and thus delineation of the exact role of any particular sequence is not straightforward. However, sites important for targeting topoisomerase II to the nucleus have been defined in the C-terminal regions of topoisomerase II enzymes from *Drosophila*, *S. cerevisiae* and *S. pombe*. These sites were defined in each case by performing a C-terminal deletion analysis so as to generate progressive truncation of the topoisomerase II enzyme. Shiozaki and Yanagida (1992), working with the *S. pombe* topoisomerase II enzyme, found that while enzymes terminating at residues 1310, 1219 and 1198 (instead of the normal 1484 residue full-length enzyme) could complement a conditional lethal *top2* mutation, the smallest of these derivatives was transported only partially to the nucleus. This suggested both that the C-terminal domain includes a signal which is necessary for efficient nuclear localization and that at least one additional nuclear localization signal must exist elsewhere in the protein. This additional site was mapped to the first 77 residues of the N-terminal ATPase domain. Elimination of this N-terminal nuclear-localization signal alone did not impair transport to the nucleus, underscoring the importance of the sequence located in the C-terminal domain.

A similar strategy was adopted by Crenshaw and Hsieh (1993) for study of the *Drosophila* enzyme. In this case, however, the sequence influencing the efficiency of nuclear localization (which was studied in *S. cerevisiae*) was not located some 265–285 residues from the C-terminus, as in the case of the fission yeast topoisomerase II, but within 60 residues of the C-terminus.

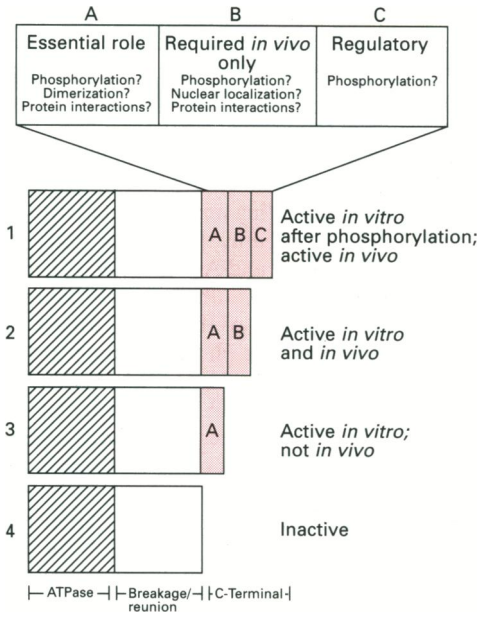

Figure 7 Roles for the C-terminal domain

The topoisomerase II protein is shown graphically (diagram 1) as comprising three equally sized domains: an N-terminal ATPase domain (cross-hatched), a central breakage/reunion domain (open box) and a C-terminal domain (light red). The C-terminal domain has been arbitrarily subdivided into three regions, designated A, B and C. An expanded map of the C-terminal domain is shown above diagram 1 in which are depicted potential roles for the three regions of the C-terminal domain. Versions of the enzyme truncated progressively from the C-terminus and lacking region C (diagram 2), regions B and C (diagram 3) and regions A, B and C (diagram 4) are shown below diagram 1. The properties of each truncated version of the protein are indicated on the right. This information is derived from studies on the *S. cerevisiae* topoisomerase II enzyme as reported by Caron et al. (1994).

However, the ability of topoisomerase II derivatives truncated by up to 240 residues to partition at least partially to the nucleus indicates that at least one other site in the *Drosophila* topoisomerase II protein is involved in directing sub-cellular localization (at least when the enzyme is expressed in budding yeast).

The third eukaryotic topoisomerase II for which analysis of nuclear localization signal sequences has been performed is that expressed by the budding yeast, *S. cerevisiae* (Caron et al., 1994). It was concluded that removal of a nuclear-localization signal positioned between Tyr-1167 and Lys-1208 contributed to the dysfunction of certain truncated derivatives. This region, which corresponds to residues Trp-1201–Thr-1265 of the *S. pombe* enzyme, implicated in nuclear localization in *S. pombe* (see above), contains a motif which closely resembles the consensus for a bipartite nuclear-localization signal sequence as defined by Dingwall and Laskey (1991).

Deletion of 221 amino acids from the C-terminus of yeast topoisomerase II has no effect on activity *in vitro* or *in vivo*, whereas several more extensive truncations of the C-terminus either render the enzyme completely inactive or lead to loss of complementing activity *in vivo* without compromising enzymatic activity *in vitro* (Caron et al., 1994). A similar finding has been made by Crenshaw and Hsieh, (1993) in studies of the *Drosophila* topoisomerase II enzyme. The 'intermediate' class of mutant proteins which are proficient *in vitro* but not *in vivo* are particularly interesting in that they are able to relax supercoiled DNA *in vivo* but are unable to decatenate DNA efficiently *in vivo*. Nevertheless, the decatenation activity *in vitro* of the purified mutant enzymes remains unimpaired. *top2* mutants expressing this class of truncated enzymes still show a high frequency of chromosomal non-disjunction (Caron et al., 1994). It is possible that due to the organization and/or the viscosity of the nuclear environment, efficient segregation of DNA molecules *in vivo* requires the interaction of other factors facilitating physical separation of the decatenated DNA molecules. This may also be necessary to preclude the establishment of futile cycles of decatenation and catenation *in vivo*.

Dimerization

Eukaryotic topoisomerase II acts as a homodimer and must therefore contain defined sequences which direct dimerization of the individual protomers. Although there is no direct evidence for the presence of a dimerization signal in the C-terminal region, there are a number of pieces of circumstantial evidence which suggest that there is likely to be one. First, a derivative of the *S. cerevisiae* enzyme, containing a deletion end-point at residue 1137 is inactive *in vitro* and *in vivo*, although this deletion does not compromise the breakage/reunion domain. In contrast, a protein with a deletion end point at residue 1166 is active *in vitro*, defining the residues between Lys-1137 and Gly-1166 as vital for enzymic activity. This region, which has been implicated in dimerization based upon primary sequence analyses (Caron and Wang, 1994), is where a number of mutations in the budding yeast *TOP2* gene conferring temperature sensitive growth have been mapped. The possible role of a leucine zipper region in the dimerization of eukaryotic topoisomerase II proteins has been discussed earlier in this review.

Phosphorylation of eukaryotic topoisomerase II enzymes

There has been considerable interest in the role of phosphorylation in modulating the action of topoisomerase II. This is primarily because phosphorylation appears to regulate not only enzymic activity, but also the responsiveness of the enzyme to inhibition by topoisomerase II-targetting drugs.

Regulation of topoisomerase II functions by phosphorylation

It has been suggested that phosphorylation plays a role both in determining specific activity and in regulating the mitotic functions of topoisomerase II. These conclusions have largely relied upon studies utilizing purified topoisomerase II proteins from lower eukaryotes. However, recent progress using mammalian systems has also been evident.

Topoisomerase II from *Drosophila* cells is a high-affinity substrate for casein kinase II *in vitro* and *in vivo* (Ackerman et al., 1985, 1988). Following phosphorylation *in vitro* by casein kinase II or protein kinase C, the specific activity of the purified *Drosophila* enzyme is stimulated 2–3-fold, apparently via an enhancement in the rate of ATP hydrolysis (DeVore et al., 1992; Corbett et al., 1992, 1993a, 1993b). These very similar effects on ATP hydrolysis and specific activity by two very different kinases imply that a common mechanism may underlie stimulation of topoisomerase II activity. This seems curious, particularly considering that casein kinase II and protein kinase C are generally thought to localize to different cellular compartments (although nuclear forms of protein kinase C have been described) and recognize distinct primary sequence motifs with no apparent similarity: serine or threonine in a general acidic context, in particular S/TXXD/E for casein kinase II; and serine or threonine in a basic context, in particular RRXXS/T for protein kinase C (where X is any amino acid).

The budding yeast topoisomerase II enzyme is also a high-affinity substrate for casein kinase II (Cardenas et al., 1992). Indeed, this kinase copurifies with topoisomerase II from both budding yeast and *Drosophila* cells (Sander et al., 1984; Cardenas et al., 1993; Bojanowski et al., 1993) (as it does from human cells also; A. M. Fry and I. D. Hickson, unpublished work). The importance of casein kinase II as a regulator of topoisomerase II activity is underscored by the observation that it is this kinase alone which is generally able to reactivate topoisomerase II previously inactivated by treatment with a general phosphatase. Thus, the *S. cerevisiae* topoisomerase II can be inactivated by alkaline phosphatase and reactivated by casein kinase II (Cardenas and Gasser, 1993; Cardenas et al., 1993), a finding paralleled by our studies using purified human topoisomerase IIα protein (A. M. Fry and I. D. Hickson, unpublished work). The topoisomerase II enzyme from mouse FM3A cells (no isozyme information was reported) can also be inactivated by alkaline phosphatase treatment and can be reactivated by a copurifying kinase. However, in this case, the kinase was reported to exhibit properties similar, but not identical, to those of casein kinase II in terms of sensitivity to both heparin and GTP (Saijo et al., 1990). In complete contrast, Shiozaki and Yanagida (1992) reported that the fission yeast topoisomerase II enzyme could not be inactivated by dephosphorylation and could find no significant role for phosphorylation in modulating enzymic activity. The possibility that phosphorylation was involved in the process of nuclear localization, was, however, suggested. Thus the budding and fission yeast enzymes appear to be regulated quite differently, although it would appear that the model set by the budding yeast enzyme is that which is conserved in many other eukaryotic organisms.

Casein kinase II is not the only kinase implicated in the regulation of eukaryotic cell topoisomerase II activity. Protein kinase C not only phosphorylates topoisomerase II in cells from humans, *Drosophila* and the sponge *Geodia cydonium*, but also increases catalytic activity by 2–3-fold (Sahyoun et al., 1986; Rottman et al., 1987; Corbett et al., 1993b).

Cell cycle regulation of phosphorylation

The phosphorylation status of topoisomerase II is regulated with respect to both cellular-growth state and cell-cycle position in eukaryotic cells. Thus, the level of topoisomerase II phosphorylation is higher in mitotic cells than in G1 cells from budding yeast, although the sites of phosphorylation are broadly similar during all cell-cycle phases, and appear to be dependent upon the activity of casein kinase II (Cardenas et al., 1992). Little or no phosphate is incorporated into the topoisomerase II enzyme in a conditional casein kinase II mutant at the non-permissive temperature. This key role for casein kinase II is emphasized by the observation that topoisomerase II and casein kinase II associate in a catalytically active complex in budding yeast cells (Bojanowski et al., 1994).

Topoisomerase II expression and phosphorylation is also cell-cycle regulated in higher eukaryotes (for example, see Kimura et al., 1994). Heck et al. (1989) showed that topoisomerase II phosphorylation is maximal during the G2/M phases of the cell cycle in chicken lymphoblastoid cells. A similar result was reported by Saijo et al. (1992) using mouse Swiss 3T3 cells, and by Burden et al. (1993) using Chinese hamster ovary cells. Thus, it seems likely that a common, if not universal, aspect of topoisomerase II regulation is the hyperphosphorylation of the enzyme during the G2 and M phases of the cell cycle.

The topoisomerase IIα and β proteins from Muntjac cells have recently been shown to be among the major chromosomal proteins recognized by the monoclonal antibody MPM-2 (Taagepera et al., 1993). The epitope for MPM-2 is conserved in a number of nuclear proteins and is dependent upon phosphorylation during or just before M-phase. Thus it appears that both topoisomerase IIα and topoisomerase IIβ are phosphorylated by a kinase that is activated at mitosis or which only encounters its substrate(s) at mitosis. Recent data indicate that at least two kinases are capable of generating immuno-reactivity with MPM-2 and that one of these is probably the mitogen-activated protein kinase (MAP kinase) (Kuang and Ashorn, 1993). The identity of the second kinase is unknown, although both kinases seem in some way to require the action of the $p34^{cdc2}$ kinase, the master controller of mitotic events. The phosphorylated epitope present in a number of antigens recognized by MPM-2 has been identified and contains an invariant (S/T)P motif with the commonest consensus being LTPLK (one letter amino acid code) (Westendorf et al., 1994). This sequence is consistent with phosphorylation either by MAP kinase or the $p34^{cdc2}$ kinase. Although both topoisomerase IIα and topoisomerase IIβ are clearly targets for a 'mitotic' kinase, little is known about the effects of phosphorylation on the activities of the different isoenzymes. Data from our laboratory indicate that both isoenzymes are multiply phosphorylated in the human cervical carcinoma cell line, HeLa, and that the sites of phosphorylation seen in each isoenzyme vary throughout the cell cycle. (N. Wells, C. Addison and I. D. Hickson, unpublished work).

Sites of phosphorylation

The precise sites of phosphorylation have not been identified unequivocally for any eukaryotic topoisomerase II enzyme, although predictions have been made in the case of the budding yeast enzyme based upon the relative mobilities of phosphorylated peptides during two-dimensional separation (Cardenas et al., 1992). All of the predicted sites map to within 350 amino acids of the C-terminus of the yeast protein. The C-terminal domain of the fission yeast topoisomerase II enzyme also appears to be a target for phosphorylation, although additional sites of phosphorylation are found in the N-terminal region of this enzyme (Shiozaki and Yanagida, 1992).

The proposal that the extreme C-terminal domain is the major target for regulatory phosphorylation of the eukaryotic topoisomerase II enzymes is hard to reconcile with the apparent dispensible nature of this region as shown by several studies utilizing C-terminally truncated versions of the enzyme (discussed above). The most plausible explanation (Cardenas and Gasser, 1993) for these apparently contradictory data is that the C-terminal domain serves a negative regulatory role which is relieved by phosphorylation. Thus, in the truncated versions of the enzyme lacking a large proportion of the C-terminal domain, no stimulatory phosphorylation is required and the enzyme is consequently fully functional *in vivo*. A similar explanation has been advanced for the effects of phosphorylation on the activity of the mammalian DNA ligase I enzyme (Prigent et al., 1992).

Phosphorylation and antineoplastic drug resistance

Phosphorylation appears to alter the susceptibility of eukaryotic topoisomerase II to inhibition by antitumour drugs. The most extensive work in this area has been perfomed by Osheroff and colleagues using the enzyme from *Drosophila* cell nuclei. Phosphorylation by either casein kinase II or protein kinase C leads to an attenuation of the effects of etoposide or *m*-AMSA in stabilizing the topoisomerase II cleavable complex. There appears to be an additive effect when the two kinases are used in

combination and this is mediated by an increase in the apparent first-order rate-constant for DNA re-ligation, the step in the catalytic cycle inhibited by antineoplastic drugs (DeVore et al., 1992).

It is possible that some of the changes that occur during adaptation to growth in the presence of topoisomerase II targeting drugs are mediated by changes in the phosphorylation status of topoisomerase II itself. For example, Takano et al. (1991) reported that topoisomerase II is 15-fold hyperphosphorylated in a human KB cell line made resistant to the growth inhibitory effects of etoposide. If these data are correlated with those of Osheroff and colleagues, it can be seen that hyperphosphorylation could be a straightforward way for cells to overcome the cytotoxic effects of topoisomerase II inhibitors. Moreover, such hyperphosphorylation, which would be expected to lead to an increase in specific activity of individual topoisomerase II molecules, may compensate for the decline in overall topoisomerase II protein level which is frequently observed in drug resistant cell lines (reviewed in Beck and Danks, 1991; Beck et al., 1993; Corbett and Osheroff, 1993; Capranico and Zunino, 1992; Fernandes et al., 1993; Pommier, 1993).

CONCLUDING REMARKS

The remarkable degree of primary sequence conservation among topoisomerase II proteins, coupled with their ubiquitous expression, attests to the biological significance of these widely studied enzymes. Indeed, the resolution of topologically constrained DNA molecules is crucial for all aspects of DNA metabolism and for cell division. The reactions catalysed by topoisomerases are mechanistically highly complex and one of the key challenges for the future will be to delineate the precise details of how DNA is transported through a protein-bound DNA gate. It is hoped that ongoing structural analyses of bacterial and eukaryotic topoisomerases will help to elucidate the molecular mechanism of this reaction. While our knowledge of the biology of bacterial and yeast topoisomerases has advanced steadily over the last decade, there is still much to be learnt about their human cell counterparts. A major unanswered question is the extent to which the human topoisomerase IIα and IIβ isozymes have evolved specialized roles. Because the human isozymes are of such importance as targets for chemotherapeutic drugs, it will be vital to study the mechanisms by which human cells control their topoisomerase II enzyme expression. If this control is shown to be mediated at the level of gene transcription, this will be feasible in the case of the human α isoenzyme, since the regulatory sequences of the topoisomerase IIα gene have been identified (Hochhauser et al., 1992). The ultimate goal would then be to maximize therapeutic intervention in those patients with tumours which fail to respond adequately to conventional therapies.

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